Automatic optimization of drug cocktails on an integrated microfluidic system

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Drug cocktails have been popular for a variety of therapies of complicated diseases. Nevertheless, it is a tediously challenging task to optimize formulations, especially using traditional methods. Hence, an automatic system capable of precise dispensing multiple drugs is of great need. Herein, a new integrated microfluidic system combined with a two-axis traverse module was developed to dispense and mix a small amount of drug combination precisely and automatically. This on-chip dispensing process could be performed with a precise and accurate manner when compared to the manual operations. The efficacy of both single and multiple drugs could be examined through the developed microfluidic system with extremely low variation of drug formulations. Analysis of cell viabilities for normal and tumor cells was also performed to verify potential drug combinations. It is envisioned that this automatic system, which is flexible to combine with standard cell analysis methods and novel drug formulation algorithm, could provide precise and high-throughput drug cocktail formulations and expedite the drug screening processes. *Published by AIP Publishing.* [http://dx.doi.org/10.1063/1.4983614]

NOMENCLATURE

CV%	coherence of variance percentage
DMSO	dimethyl sulfoxide
Doc	docetaxel
Dox	doxorubicin
EMV	electromagnetic valve
Etop	etoposide
FBS	fetal bovine serum
FSC	feedback system control
IC50	half maximal inhibitory concentration
MEF	mouse embryonic fibroblasts
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	optical density

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PDMS polydimethylsiloxane
Rap rapamycin
Vin vincristine
5-FU 5-fluorouracil

I. INTRODUCTION

Combination of different therapeutic agents has been widely exploited to enhance single-drug efficacy and lower side effects in clinical applications. These drug formulations, as well as drug cocktails, are becoming the standards of treatment for a number of complicated diseases, such as tumors, infectious diseases, neurodegenerative diseases, and metabolic syndromes.[1,2] Optimization of drug cocktail formulation by trial-and-error is a labor-intensive and time-consuming task due to the considerable amounts of combinations. For instance, finding the optimal conditions for six different drugs at 10 different concentrations requires 1 million potential tests. Furthermore, a complete search for the most optimal combination in the *in-vivo* or clinical tests is not practical even for combinations of only 2 to 3 drugs. Hence, a new approach to simplify the optimization progresses is needed. Recently, a feedback system control (FSC) scheme[3,4] was reported which could rapidly identify the best combination of drug dosages in fewer tests and therefore bypass the need to test all the potential test trials.[5,6] The approach can save several orders of magnitude in terms of experimental efforts and cost and has been used in a variety of applications, including inhibition agents of infectious diseases, regulation agents on stem cells, anti-cancer drugs, and the identification of the multiple compounds in herbal medicine.[7–11]

Although FSC avoids tremendous efforts to test all potential trials, a few iterative cycles of close-loop optimization are still required, indicating that tedious drug combination processes using a small amount of drugs are still inevitable. A well-trained technician is required to administrate considerable amounts of drugs with different concentrations such that intensive labors and time are inevitable, which may also lead to unexpected bias. Therefore, a new system for automatic drug dispensing is greatly needed.

Recently, microfluidic technologies which integrate multiple functional components (such as micro-pumps, micro-valves, and micro-mixers) for biomedical or chemical analysis have emerged as a promising tool. It has become available to perform several crucial operations on a single, integrated microfluidic system including sample pretreatment, transportation, mixing, reaction, separation, and detection.[12,13] Therefore, it may offer several advantages over their large-scale counterparts, including a significant decrease in sample and reagent consumption, quicker reaction times, high sensitivity, high throughput, portability, low power consumption, and low cost when performed on an automated, integrated, and miniaturized disposable chip.[13]

Microfluidic devices for accurate and precise liquid delivery have been extensively investigated in literatures.[14–18] Among them, the most popular methods for liquid sample delivery in microfluidic devices are mechanical or membrane-based micro-pumps, such as piezoelectric, electrostatic, electromagnetic, pneumatic, and thermo-pneumatic approaches.[14–18] For instance, polydimethylsiloxane (PDMS)-based pneumatically driven micro-pumps have been widely used for microfluidic devices to transport reagent solutions, especially for biochemical applications.[19–22] In addition, several advantages of PDMS, such as high biocompatibility, high deformability, and relatively low-cost, make the PDMS-based microfluidic devices popular. For example, previous studies in our group have reported that these pneumatically driven microfluidic devices are capable of precise sampling.[23,24] Nevertheless, this micro-dispenser is only subjected to one sample, which may not be suitable for drug cocktails.

Drug screening on microfluidic systems has attracted increasing interest since it provides several advantages described above. Most studies combine cell culture techniques for directly detecting biological responses or mimicking micro-environments.[25–28] These studies successfully miniaturize and simplify the drug screening system. However, there are also several disadvantages involved with these microfluidic systems. For example, they are specific for single purpose and unable to combine with a regular standard cell assay.[27] More importantly, none of

them demonstrated that more than three multiple drug combinations could be precisely dispensed, vigorously mixed, and delivered to cell cultures by using the microfluidic devices. Thus, a simple drug-dispenser capable of more than three different drugs flexibly combined with a regular cell analysis assay on commercial culture plates is investigated here.

In this work, a new micro-dispenser device was designed herein to automatically administrate different drugs. In this microfluidic device, there are several pneumatically driven micro-pumps, normally closed micro-valves, and micro-chambers for loading drugs and reagents. The micro-pumps could serve for dispensing and mixing, and normally closed micro-valves block the contamination of samples. Up to six different drugs preloaded in the micro-chambers could be automatically and simultaneously dispensed and mixed, which can shorten the operation time and enhance the throughput. For dispensing the drug mixture to each cell culture on a standard plate, the microfluidic device was set on a two-axis traverse module, which could be traversed to any location such that it could be flexible for any kind of standard cell assay plate. Experimental results showed that the developed integrated microfluidic system is capable of accurate dispensing and effective mixing. Most importantly, by using the developed automatic microfluidic platform, experimental results showed relatively low bias and the operation time was significantly reduced by approximately 20%. In addition, it can be designed and programmed for both single and multiple drug administration. The present system was designed for a standard 96-well cell culture assay; furthermore, with the advantage of microfluidic techniques, the usage of drugs and reagents could be greatly decreased in the near future. In conclusion, this system may be promising to be combined with the FSC algorithm to expedite the drug cocktail screening.

II. MATERIALS AND METHODS

A. Experimental procedures

The protocol for automatically dispensing different drug combinations to cell cultures is illustrated in Fig. 1 and supplementary material Figure 1. Briefly, six different drugs (for combination tests) or single drug with different concentrations (for half maximal inhibitory concentration, IC50, test) were pre-loaded in the drug loading chambers on the micro-dispenser. Then, the specified volumes of drug solutions were automatically injected into the central chamber by

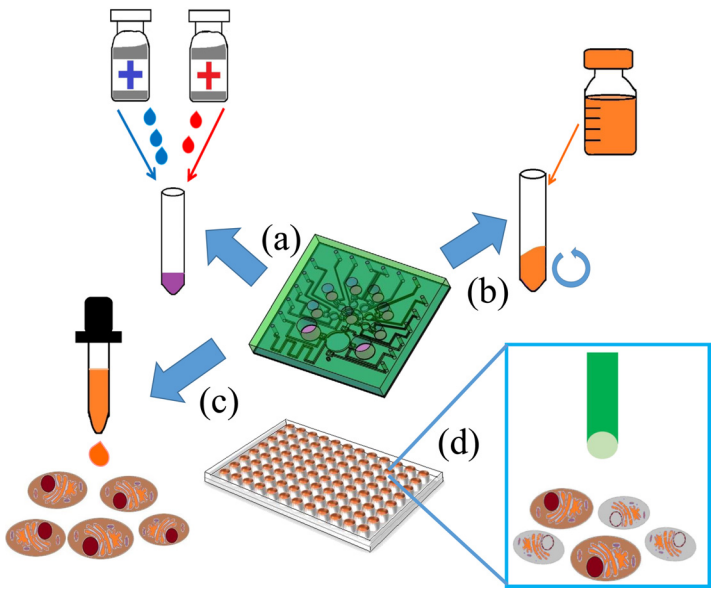

FIG. 1. A schematic illustration for administrating one drug combination, including (a) drug dispensing of two or more different drugs, (b) drug mixing with drug combinations, serum, and culture medium, (c) drug injection from the micro-dispenser to cell cultures, and (d) cell analysis after incubation with the drug mixture.

activating the pneumatically driven dispensing micro-pumps. The resulting drug combinations were subsequently mixed with fetal bovine serum (FBS) by activating the pneumatically driven central injection micro-pump for 30 min at a frequency of 0.25 Hz. The mixtures were then added with culture medium, briefly mixed for 10 s with 0.25 Hz, and vertically injected with desired volumes into the individual wells containing cell cultures on a 96-well microtiter plate. The X-Y positions of the micro-dispenser and 96-well plate were precisely controlled by the positioning module to inject the drug mixture from one well to another. Note that all the steps of the protocol were automated in the integrated microfluidic system, which consists of a microfluidic chip, an electromagnetic valve (EMV) controller, and a two-axis traverse module. Finally, the cells treated with different combinations of drugs were analyzed by using a cell viability assay to examine the drug toxicity.

B. Chip design and fabrication process

A micro-dispenser was designed and fabricated for the purpose of automatic drug dispensing and mixing as described above. As shown in Fig. 2(a), two different sizes of pneumatically driven micro-pumps were used in the micro-dispenser. One was designated as micro-dispensing pump to transport the individual drug samples. Note that six identical dispensing micro-pumps were designed to be incorporated with six storage chambers and six micro-valves such that six drugs could be dispensed separately to the central chamber. Another micro-pump was designated as the injection micro-pump with dual functions to mix drugs/serum/medium and to inject the mixtures from the microfluidic device to the cell cultures. In addition to the central chamber, two storage chambers were designed for FBS and culture medium, respectively.

The micro-dispenser was made of PDMS (Sylgad 184 A/B, Dow Corning Corp., USA) and replicated by molding from a polymethylmethacrylate master template, which was fabricated by a computer-numerical-control machining process (EGX-400, Roland Inc., Japan). The dimensions (length × width) of the micro-dispenser were measured to be 50.0 mm × 50.0 mm (Fig. 2(b)), which are much smaller than those of a standard 96-well microtiter plate. As shown in Fig. 2(c), the microfluidic device consists of three PDMS layers and one glass substrate (G-Tech Optoelectronics Corp., Taiwan). The top thick PMDS layer (10 mm) was designated as an air-control layer and the middle thin PDMS layer (0.4 mm) was designated as a liquid-channel layer while an additional PDMS layer (3 mm) assembled on the back side of the glass substrate was designated as a nozzle layer. Notably, this nozzle layer contained a 0.6-mm-diameter vertical channel, which was designed for changing the liquid route from the horizontal direction to the vertical direction. The outer surface of the nozzle layer was treated with hydrophobic materials (NeverWet, Rust-Oleum, USA) to avoid accumulation of liquid drops on the surface.[29] All the PDMS layers and the glass substrate were bonded together to form a sealed microfluidic chip using an oxygen plasma method.[30] Drug molecules may be absorbed into PDMS;[31] therefore, the micro-dispensers used in the present study were blocked with 0.2% (w/v) bovine serum albumin in phosphate buffer solution prior to usage.

C. Operation of the micro-dispenser and 2-axis traverse module

An EMV controller equipped with 18 EMVs (S070M-SBG-32, SMC Inc., Japan) connected with a compact air supplier containing an air compressor (TC-10, Sun Mines Electrics Co. Ltd., Taiwan) and a vacuum pump (DC-16 V, Uni-crown Co. Ltd., Taiwan) was used to activate our micro-dispenser with programmed and designed processes. The detailed operating principle could be found in our previous work.[32] Supplementary material Figure 2 illustrates the operating principle of the micro-dispenser. The major difference between the micro-dispenser and our previous devices is that the present micro device equipped with an additional nozzle layer is capable of the micro-injector function, which could inject liquids vertically to form droplets. Briefly, these normally close valves and micro-pumps on the microfluidic device could trigger the transportation of drug solutions and reagents within different chambers or liquid injection from the micro-dispenser to the cell culture plate by switching applied negative or positive

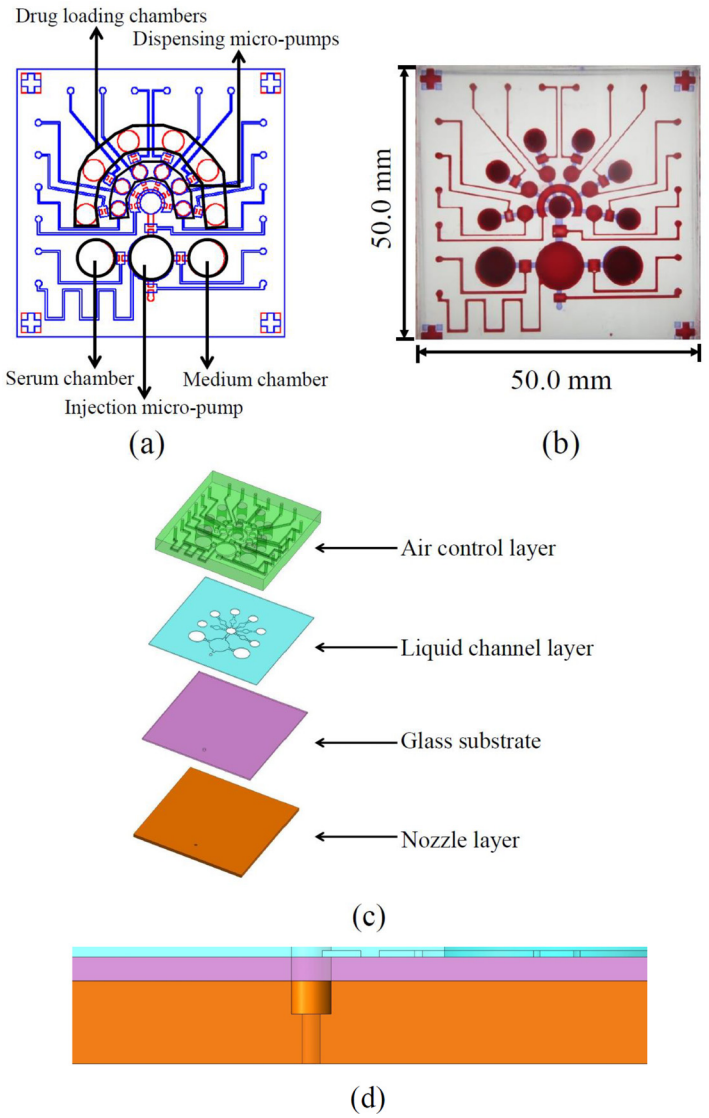

FIG. 2. (a) An integrated micro-dispenser equipped with multiple micro-devices, including six drug loading chambers, six dispensing micro-pumps, two large loading chambers for serum or medium, and an injection micro-pump. (b) A photograph of the integrated micro-dispenser. (c) An exploded view of the micro-dispenser, which was composed of an air control layer, a liquid channel layer, a glass substrate, and an additional nozzle layer. (d) The cross-sectional view of the nozzle, which was composed of a liquid channel layer, the glass substrate with a hole, and the nozzle layer.

gauge pressures to uplift or release PDMS membranes. Therefore, a precise amount of the drugs could be dispensed to the corresponding chambers.

A stepping motor controller (08TMC-2 U, Unice E-O Service Inc., Taiwan) was used to control the 2-axis traverse module (08UMT-100 and 08UMT-50, Unice E-O Service Inc., Taiwan) such that the micro-dispenser could be moved precisely to any location of the 96-well cell culture plate (Supplementary material Fig. 3). In order to hold the micro-dispenser and the culture plate firmly, specifically designed frame stages with hanging arms were fabricated by a 3 D printer (Mbot, Magicfirm, USA). The frame stages were then fixed on the 2-axis traverse module and the gap between the micro-dispenser and the culture plate was fixed at 5 mm by using these hanging arms. The moving speed of the traverse module was controlled at 10 mm s^{-1} (The resolution is 0.00125 mm and the triggering signals provided by the controller are 8000 pulses s^{-1}.) With this approach, the culture medium and drug solutions would not be spilt out of the wells. The movement of the traverse module was programmed and synchronized

with the processes of the EMV controller. Therefore, the drug solutions could be dispensed, mixed, and injected into cell cultures with an automated manner.

D. Measurement of injection volume and mixing index

In the present study, a pneumatically driven micro-pump has been developed to precisely transport desired volume of liquids, which was revised from our previous works.[23,24,32] Briefly, the micro-pumps were connected to an EMV that was regulated to an EMV control. It could be activated by applying positive or negative gauge pressure from a vacuum pump or a compressor. For instance, the negative gauge pressure (vacuum) provided a suction force to uplift the PDMS membrane of the thin liquid-channel layer for liquid transportation. In order to characterize the performance of the micro-pumps, the injection volumes were measured under different vacuum gauge pressures. After the transporting processes, the injected volumes of liquids in the chambers were measured by calculating the weights of the liquid.

Notably, the pneumatically driven PDMS layers also provided the mixing function for mixing drugs and FBS. In order to examine the mixing efficiency of the micro-pump, the mixing index under the applied driving frequency was measured by using a charge-coupled device camera to acquire optical images as previously described.[32] Here, the mixing of 50 μl of water and colored ink was measured. These captured images were subsequently analyzed using digital imaging software to evaluate the mixing index.

The accuracy and precision of liquid injection using the micro-injector were further evaluated. Random eight positions on a 96-well rack with the same dimensions as those of a standard 96-well culture plate were chosen. Each position was placed with an empty microcentrifuge tube. The diameter of a microcentrifuge tube ($\sim$5.45 mm) is much smaller than that of a well on a 96-well plate ($\sim$6.85 mm). Hence, the liquid injected into the microcentrifuge tubes was measured to simulate the injection process on a 96-well plate. After injection using the microfluidic system, the volumes of liquids in the targeting tubes were measured. Manual operations were also performed for comparison by using a regular 200-μl pipette. The accuracy and precision of the injected volumes were then calculated accordingly.

E. Anti-cancer drugs and cell cultures

Six different drugs, including 5-fluorouracil (5-FU), etoposide (Etop), doxorubicin (Dox), vincristine (Vin), docetaxel (Doc), and rapamycin (Rap), were purchased from Sigma Aldrich Inc., USA, dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and stored in $-80\,^{\circ}$C prior to usage. The stock solutions of drugs were diluted with DMSO at indicated concentrations for cell viability tests. It was known that Dox was inherently fluorescent and light-absorbent.[33] This special characteristic could be utilized to measure the relative concentration. Here, we only utilized the light-absorbent property and we found that the absorbent peak of visible light spectra was located at 480 nm, which was consistent with that described previously.[36] In order to examine the accuracy and precision of dispensing two different drugs by the micro-dispenser, the absorbance spectra of 5-FU and Dox in DMSO after being dispensed by using the developed micro-dispenser were detected by using a spectrophotometer (Nanophotometer™ Pearl; IMPLEN, Germany).

In this study, wild-type mouse embryonic fibroblasts (wt-MEF; CRL-2991™) and mouse leukemia cells (WEHI-231; CRL-1703™), which were obtained from American Type Culture Collection (ATCC; USA), were used to test the efficacy of single drug and drug cocktails The wt-MEF cells and WEHI-231 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% FBS, 100 UI/mL penicillin-streptomycin (Invitrogen, USA), and additional 0.05 mM of 2-mercaptoethanol specific for WEHI-231 cells. All cell lines were incubated at 37 °C in 5% CO_2 according to the instructions provided by ATCC. For cell viability assay of wt-MEF cells, 1×10^4 cells in 100 μl of culture medium were seeded in a 96-well microtiter plate. In the same experiment for each single microtiter plate, 5×10^4, 2.5×10^4, 1×10^4, 7.5×10^3, 5×10^3, 2.5×10^3, and 1×10^3 wt-MEF cells in 100 μl of culture medium were also seeded for obtaining standard curves of cell numbers. WEHI-231 cells, which were normally suspended in medium, were especially seeded and attached on poly-L-lysine-coated culture plates.

These plates were added with 25 μl of 0.1 mg/ml poly-L-lysine solution (Sigma Aldrich, USA) per well. After 15 min of incubation, the solutions were removed. The wells of microtiter plates were subsequently rinsed with 25 μl of double-distilled water and air dried for at least 1 hour before they were seeded with cells. Seventy-five thousands of WEHI-231 cells in 100 μl of culture medium were then seeded for drug treatment test, whereas 1×10^5, 7.5×10^4, 5×10^4, 2.5×10^4, 1×10^4, 7.5×10^3, and 5×10^3 WEHI-231 cells in 100 μl of culture medium were seeded for obtaining standard curves of cell numbers. Approximately 12 h after all the cells were attached on the plates, these cells were mock treated (DMSO only for standard curves) or treated with single drug in different concentrations or multiple drug mixtures by using the micro-dispenser or manual operations. In order to compensate the operation time for synchronizing the drug-treating periods, the subsequent steps for each cell culture were processed with an approximately 10 s interval. Twenty hours after drug treatments, the cells were incubated with 0.5 mg/ml of 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for additional 4 h. The resulting cells were subsequently added with 100 μl of DMSO, and the optical density (OD) at a wavelength of 570 nm was measured by using a microtiter plate reader (Multiskan EX, Thermo Fisher Scientific, USA). The cell viabilities after drug treatment were then calculated based on the calibration curves obtained by the MTT assay. Note that all experiments were performed in triplicate samples for at least two times independently. The IC50 values of drug-response curve fitting were finally calculated by the Graph Prism software (GraphPad Software, Inc., USA). The two-tailed student's t test was calculated by using the Microsoft Excel software and used to perform statistical analyses in the present study, and $P < 0.05$ was considered as statistically significant.

III. RESULTS AND DISCUSSION

A. Characterization of the micro-dispenser

The major purpose of this study is to dispense a specific volume of drug solutions accurately and precisely for the subsequent cell-level tests such that an optimal combination of drug cocktails could be explored. Therefore, the performance of the micro-dispenser was first characterized. The relationship between the pumping volume and the applied gauge pressure was measured and is shown in Fig. 3(a). The transported volume was observed to increase along with the applied gauge pressure, demonstrating that the desired volume of injected liquids driven by

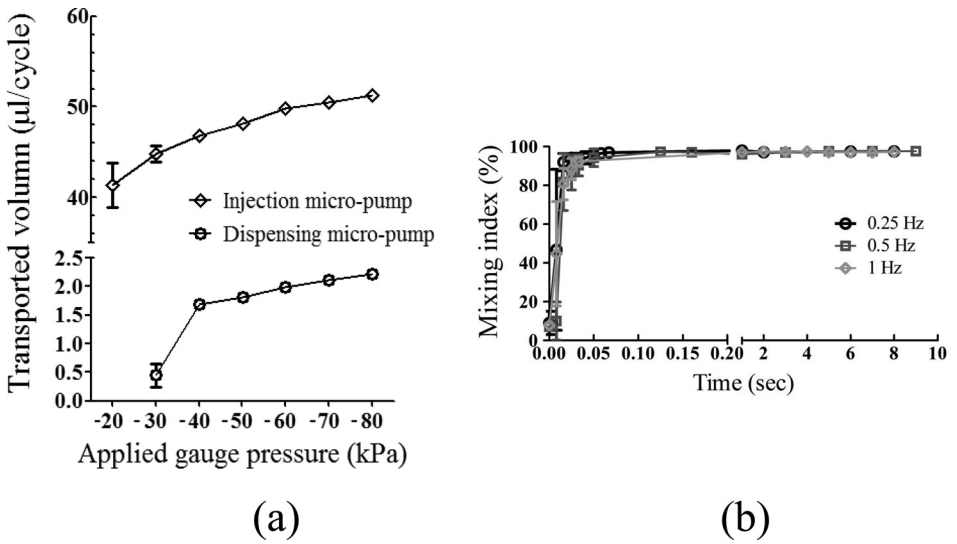

FIG. 3. (a) Measurement of transported volume of pneumatically driven micro-dispenser. Two different micro-pumps on the micro-dispenser, including an injection micro-pump and a dispensing micro-pump, were tested. The injected volumes of double-distilled water were measured under different applied gauge pressures. (b) The mixing index of the micro-dispenser at three driving frequencies (0.25, 0.5, and 1 Hz, respectively). A mixing index of 97% was achieved within 0.1 s under a gauge pressure of −80 kPa.

both dispensing and injection micro-pumps could be precisely fine-tuned. Note that the dispensing volumes were varied from 0.5 to 2.0 μl, which were suitable for drug dispensing in the subsequent experiments. In addition, the injection volume was 50 μl while an applied gauge pressure was set at −60 kPa, which was designated for the cell cultures on a 96-well plate.

Furthermore, the accuracy and precision of injection volumes provided by the micro-dispenser while combined with the 2 axis traverse module were explored. Table I lists the operation errors using our micro-dispenser automatically or a pipette manually. The accuracy of the micro-dispenser in different positions was measured to range from ±0.04% to ±1.09%, with a mean systemic error of ±0.17%, which was much less than that using the pipette manually (±0.75%). Similarly, the precision of the micro-dispenser in different positions was measured to range from 0.34% to 2. 97% with a total random error of 1.59%, which was comparable to that using the pipette manually (1.56%). Based on these results, this integrated microfluidic chip combined with the 2-axis traverse module could inject the drug solutions to cell cultures on a 96-well plate more accurately and precisely than the manual pipetting operations.

Efficient mixing plays an important role in drug combinations, because weak drug mixing may lead to drug precipitation, therefore lowing drug efficacy.[34] Besides the transportation function, there was also a mixing function on the pneumatically driven micro-pump of the microfluidic chip.[32] The mixing indexes under various driving frequencies of the injection micro-pump on the micro-dispenser are shown in Fig. 3(b), demonstrating that the mixing index could achieve 96% within 0.1 s under driving frequencies of 0.25, 0.5, or 1 Hz, respectively. It means that sufficient mixing could be achieved within a short period of time. Following the previous protocols in which the drugs were needed to mix with serum thoroughly, the drug solutions would be kept in gentle mixing for 30 min to avoid possible drug precipitation before being injected to cell cultures. In order to prevent the bubbles occurring in the drug solutions with serum and culture medium during the whole mixing process, only a low driving frequency (0.25 Hz) was applied for gentle mixing and avoiding precipitation.

B. Single drug administrated by the integrated microfluidic system

Six different anti-tumor drugs, including 5-FU, Etop, Dox, Vin, Doc, and Rap, were used in this study. All of them were generic drugs and widely used for cancer treatment. Dox has inherent red color and could be detected using a spectrophotometer.[33] With this feature, Dox and another drug, 5-FU, were first selected and used to verify the feasibility of the developed microfluidic system to dispense two kinds of drugs with desired ratios. Figure 4(a) shows that the absorbance of Dox at a peak of 480 nm, while that of 5-FU was not detected. Therefore, the concentration of Dox in mixed solutions with different ratios (ranging from 5.0 to 0.2) could be determined accordingly (Fig. 4(b)). The results indicated that the micro-dispenser has satisfactory quantitative performance to mix two different drugs accurately and precisely with an automatic manner. Note that three repeated measurements were performed and the coherence of variance percentage (CV%) was within 4.1%, whereas the CV% was about 5.6% with manual operations (data not shown).

TABLE I. Operation errors using the micro-dispenser automatically on 8 random positions or a regular pipette manually.

	Micro-dispenser									
Position	#1	#2	#3	#4	#5	#6	#7	#8	Mean	Pipette
Systemic error[a]	±1.03	±0.04	±0.40	±0.06	±0.23	±0.32	±1.09	±0.19	±0.17	±0.75
Random error[b]	2.18	1.17	0.34	0.35	2.28	0.47	2.97	0.81	1.59	1.56

[a]Percentage of [(mean of measured volume – desired volume) ÷ desired volume].
[b]Percentage of (standard deviation of measured volume ÷ mean of measured volume).

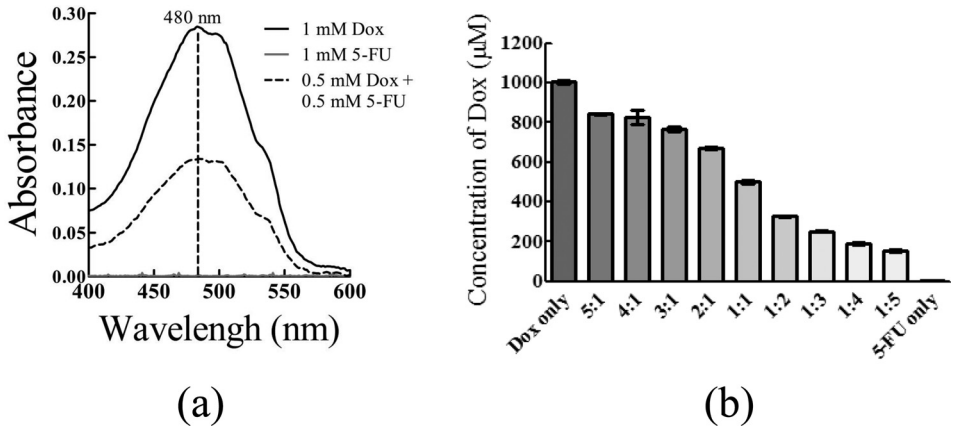

FIG. 4. Dispensing tests of the micro-dispenser using two anti-tumor drugs (Dox, 1 mM and 5-FU, 1 mM). The absorbance spectra are shown in (a) with a peak at 480 nm. These two drugs were dispensed with indicated ratios on chips, and the concentrations of Dox are shown in (b).

To investigate the drug efficacy of these six drugs individually, different concentrations of single drug were administrated to mouse normal and tumor cells for testing cytotoxicity by using our micro-dispenser (on chip) or manual operations (on bench). Exposure of cells to each drug caused a concentration-dependent cytotoxicity, with very similar kinetic curves between on-chip and on-bench methods (Fig. 5). The IC50 values of 5-FU, Etop, Dox, Vin, Doc, and Rap on mouse normal cells (MEF) using on-chip protocols were approximately >100, 9.38, 0.27, 0.90, 3.41, and >100 μM, respectively, which were very close to those using on-bench methods (>100, 7.40, 0.26, 1.21, 7.85, and >100 μM). In addition, the IC50 values of each single drug on mouse leukemia cells (WEHI) using on-chip processes (14.17, 0.68, 0.64, 0.25, 1.85, and 61.73 μM for 5-FU, Etop, Dox, Vin, Doc, and Rap, respectively) were also comparable to those using on-bench manipulations (14.57, 0.65, 0.57, 0.18, 2.03, and 33.69 μM for 5-FU, Etop, Dox, Vin, Doc, and Rap, respectively). With the automatic dispensing methods, the drug efficacy was measured and comparable to the one performed by manual operations. It was noteworthy that the variation for the experiments performed on chip was smaller than that performed on chip. The CV% of survival rates using MEF on chip was measured to be 5.82%, while that using MEF on bench was 9.61%. Furthermore, the CV% of survival rates using WEHI cells on chip was measured to be 5.43%, which was smaller than that using WEHI cells on bench (14.37%). Interestingly, the mouse leukemia cells were slightly more susceptible to these drugs (except Dox) than the normal cells. However, in order to achieve high cytotoxicity on the leukemia cells by using a single drug, high concentrations are required, while the normal cells were also vulnerable with low viabilities (Fig. 5). These results suggested that not only did each single drug with high concentrations inhibit the tumor cell proliferation, but it also killed normal cells, which should be taken into consideration when using these chemical drugs.

C. Drug combination treatment

Since the micro-dispenser could administrate the single drug with different concentrations, six drugs with different combinations administrated by the micro-dispenser were also evaluated. The drug concentrations in different optimized concentrations are shown in supplementary material Table S1. DMSO, which was used as the drug solvent, was regarded to be harmful to cell culture and would cause cell death.[35] Therefore, a water-treated control group was also demonstrated. It is worth noting that DMSO, which was used as the drug solvent, was regarded to be harmful to cell culture and could cause cell death. The usage volume of DMSO could increase along with the complicity of drug combinations. Therefore, a water-treated control group should be also demonstrated, and the cell survival rates were nearly 120% when

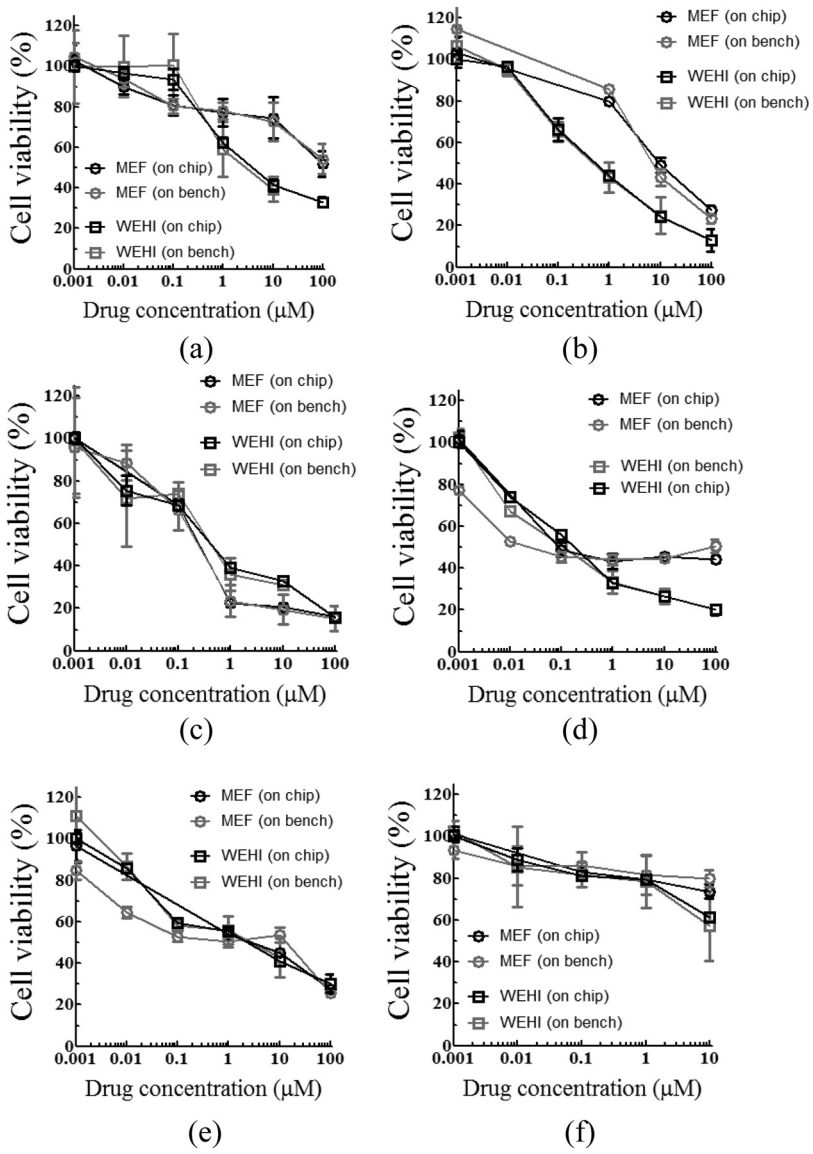

FIG. 5. The cell viability treated with single anti-tumor drugs by using the conventional dispensing methods (on bench) or the micro-dispenser (on chip). Mouse embryonic fibroblasts (MEF) or leukemia cells (WEHI) were treated with 5-fluorouracil (a), etoposide (b), doxorubicin (c), vincristine (d), docetaxel (e), or rapamycin (f) under indicated concentrations and the survival rates are shown.

compared to the DMSO-treated group (Fig. 6). Notably, the administrated concentrations of each drug were less than the IC50 values of these drugs in mouse normal cells; the concentrations of some drugs were slightly less than or close to the IC50 values of these drugs on mouse leukemia cells. Figure 6(a) demonstrates the cell viabilities with different drug combinations using the automatic administrations by using the micro-dispenser. All the drug combinations led to lower cell viabilities on leukemia cells than those on normal cells, with significant differences ($P < 0.05$, by student's t test). Similar results were also observed using on-bench methods (Fig. 6(b)). Interestingly, the variations within each group by using on-chip methods were less than those using on-bench methods (Fig. 6), which were consistent with our observations, as shown in Table I. The CV% of survival rates using MEF or WEHI cells on chip was 1.91% and 1.80%, respectively. The CV% of survival rates using MEF or WEHI cells on bench was 4.00% and 4.73%, respectively. According to the experimental results, the micro-dispenser was also capable of dispensing different drugs with various combinations to cell cultures

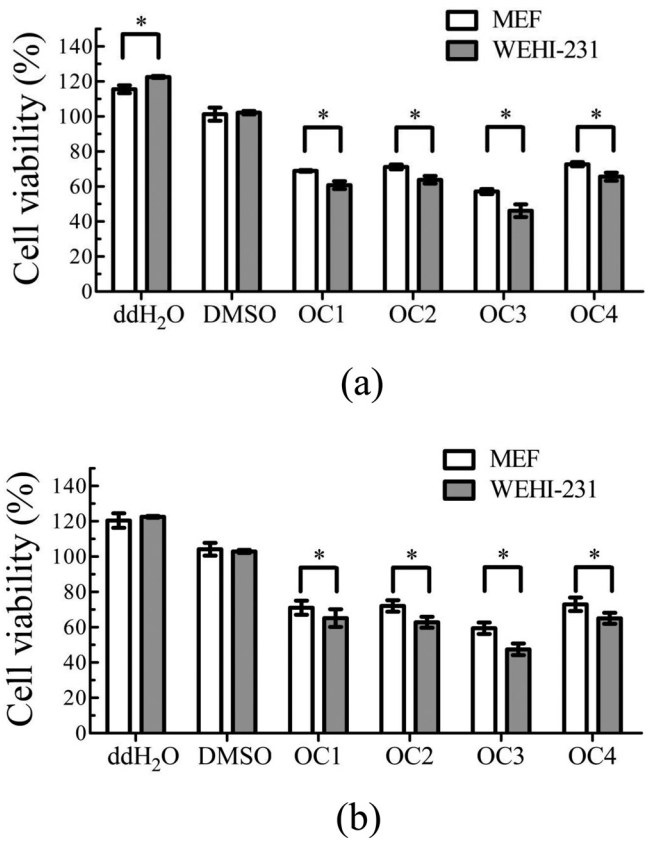

FIG. 6. The cell viability treated with different combinations of six kinds of anti-tumor drugs on the microfluidic chip (a) or on bench (b). MEF or WEHI-231 cells were administrated with ddH_2O, DMSO, or different combinations (OC1–4) of drugs. *, $P < 0.05$, student's t test.

automatically and precisely with low bias. In addition, manual operations to formulate and mix multiple drugs with a pipette were sequential and relatively time-consuming, while these procedures could be performed simultaneously by our present micro-dispenser. It was concluded that the micro-dispenser could reduce 20% of total drug dispensing time (∼2.4 h) when compared to manual operations (∼3 h).

IV. CONCLUSIONS

In this study, an integrated microfluidic system has been demonstrated to be capable of dispensing up to 6 different drugs, mixing them, and injecting the mixture to the cell cultures on 96-well plates. While incorporated with a 2-axis traverse module, all the drug dispensing processes could be programmed. This system may avoid tedious manual operations and potential bias. Indeed, our results demonstrated that comparably low bias and desired administration volumes of liquids, which was based on precisely controlled pneumatically driven micropumps.[23,24] Because the procedures of the cell culture and cell analysis were independent from those of drug dispensing and mixing, it would potentially avoid the cross-contaminations between the microfluidic devices and cell cultures. Most importantly, the micro-dispenser worked separately from cell cultures, indicating that one micro-dispenser was capable of providing several different combinations to different cell cultures on several plates during one turn of testing. In addition, the accuracy and precision by using the developed integrated microfluidic system were superior to those of manual operations. In addition to different single drug tests, the micro-dispenser could also perform the multiple drug combination tests, and the performance was superior to the manual process using a commercial pipette. The present system was demonstrated for a standard cytotoxicity assay on a 96-well plate, and the

reagent-consuming volumes with the micro-dispenser were the same as the traditional methods. However, with the advantage of microfluidic techniques, the usage of the drugs and reagents could be considerably reduced when combined with other novel cell detection methods (e.g., a real-time cell imaging microscope system; Incell 6000). Therefore, the developed automatic microfluidic system could be able to combine with an algorithm, such as FSC,[3,4] and novel cell analysis systems to expedite screening of drug combinations in the future.

SUPPLEMENTARY MATERIAL

See supplementary material for schematic illustration for administrating one drug combination performed on the developed microfluidic chip, operating principles of the micro injection device, the two-axis traverse module containing the frame stages with hanging arms, and the concentrations of each drug in different combinations.

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[1]C. L. Sawyers, Nature **449**, 993 (2007).
[2]J. Lehar, A. S. Krueger, W. Avery, A. M. Heilbut, L. M. Johansen, E. R. Price, R. J. Rickles, G. F. Short III, J. E. Staunton, X. Jin, M. S. Lee, G. R. Zimmermann, and A. A. Borisy, Nat. Biotechnol. **27**, 659 (2009).
[3]P. K. Wong, F. Yu, A. Shahangian, G. Cheng, R. Sun, and C. M. Ho, Proc. Natl. Acad. Sci. U. S. A. **105**, 5105 (2008).
[4]P. Nowak-Sliwinska, A. Weiss, X. Ding, P. J. Dyson, H. van den Bergh, A. W. Griffioen, and C.-M. Ho, Nat. Protocols **11**, 302 (2016).
[5]F. Wei, B. Bai, and C.-M. Ho, Biosens. Bioelectron. **30**, 174 (2011).
[6]C.-P. Sun, T. Usui, F. Yu, I. Al-Shyoukh, J. Shamma, S. Ren, and C.-M. Ho, Integr. Biol. **1**, 123 (2009).
[7]X. T. Ding, D. J. Sanchez, A. Shahangian, I. Al-Shyoukh, G. H. Cheng, and C. M. Ho, Int. J. Nanomed. **7**, 2281 (2012).
[8]Y. Honda, X. Ding, F. Mussano, A. Wiberg, C. M. Ho, and I. Nishimura, Sci. Rep. **3**, 3420 (2013).
[9]H. Tsutsui, B. Valamehr, A. Hindoyan, R. Qiao, X. Ding, S. Guo, O. N. Witte, X. Liu, C.-M. Ho, and H. Wu, Nat. Commun. **2**, 167 (2011).
[10]A. Weiss, R. H. Berndsen, X. Ding, C. M. Ho, P. J. Dyson, H. van den Bergh, A. W. Griffioen, and P. Nowak-Sliwinska, Sci. Rep. **5**, 14508 (2015).
[11]H. Yu, W. L. Zhang, X. Ding, K. Y. Z. Zheng, C.-M. Ho, K. W. K. Tsim, and Y.-K. Lee, Evidence-Based Complementary Altern. Med. **2013**, 541436.
[12]P. A. Auroux, D. Iossifidis, D. R. Reyes, and A. Manz, Anal. Chem. **74**, 2637 (2002).
[13]G. B. Wisdom, A. Wochner, M. Menger, D. Orgel, B. Cechet, M. Rimmele, and V. A. Erdmann, Clin. Chem. **22**, 1243 (1976).
[14]T. Bourouina, A. Bosseboeuf, and J. P. Grandchamp, J. Micromech. Microeng. **7**, 186 (1997).
[15]O. C. Jeong and S. S. Yang, Sens. Actuators A **83**, 249 (2000).
[16]D. J. Laser and J. G. Santiago, J. Micromech. Microeng. **14**, R35 (2004).
[17]H. T. G. Vanlintel, F. C. M. Vandepol, and S. Bouwstra, Sens. Actuators **15**, 153 (1988).
[18]C. Yamahata, F. Lacharme, and M. A. M. Gijs, Microelectron. Eng. **78–79**, 132 (2005).
[19]C. M. Chang, W. H. Chang, C. H. Wang, J. H. Wang, J. D. Mai, and G. B. Lee, Lab Chip **13**, 1225 (2013).
[20]E. Kim, Y. N. Xia, and G. M. Whitesides, Nature **376**, 581 (1995).
[21]C. C. Lin, J. L. Hsu, and G. B. Lee, Microfluid. Nanofluid. **10**, 481 (2011).
[22]C. H. Weng, C. J. Huang, and G. B. Lee, Sensors **12**, 9514 (2012).
[23]C. W. Huang, S. B. Huang, and G. B. Lee, J. Micromech. Microeng. **18**, 35004 (2008).
[24]S. B. Huang and G. B. Lee, J. Micromech. Microeng. **19**, 35027 (2009).
[25]P. T. Kumar, K. Vriens, M. Cornaglia, M. Gijs, T. Kokalj, K. Thevissen, A. Geeraerd, B. P. Cammue, R. Puers, and J. Lammertyn, Lab Chip **15**, 1852 (2015).
[26]A. Weltin, K. Slotwinski, J. Kieninger, I. Moser, G. Jobst, M. Wego, R. Ehret, and G. A. Urban, Lab Chip **14**, 138 (2014).
[27]Q. Wu, D. Gao, J. T. Wei, F. Jin, W. Y. Xie, Y. Y. Jiang, and H. X. Liu, Chem. Commun. **50**, 2762 (2014).
[28]Y. Q. Zhang, W. J. Zhang, and L. D. Qin, Angew Chem. Int. Ed. **53**, 2344– 2348 (2014).
[29]R. Gupta, V. Vaikuntanathan, and D. Sivakumar, Colloid Surf. A **500**, 45 (2016).
[30]J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. K. Wu, O. J. A. Schueller, and G. M. Whitesides, Electrophoresis **21**, 27 (2000).
[31]M. W. Toepke and D. J. Beebe, Lab Chip **6**, 1484 (2006).
[32]C. H. Weng, K. Y. Lien, S. Y. Yang, and G. B. Lee, Microfluid. Nanofluid. **10**, 301 (2011).

[33]A. Z. Wang, R. Langer, and O. C. Farokhzad, in *Annual Review of Medicine*, edited by C. T. Caskey, C. P. Austin, and J. A. Hoxie (Annual Reviews, 2012), Vol. 63, pp. 185–198.
[34]B. A. Hoener and L. Z. Benet, in *Drugs and the Pharmaceutical Sciences: Modern Pharmaceutics*, edited by G. S. Banker and C. T. Rhodes (Marcel Dekker, New York, 2002), Vol. 121, pp. 93–117.
[35]J. Galvao, B. Davis, M. Tilley, E. Normando, M. R. Duchen, and M. F. Cordeiro, FASEB J. **28**, 1317 (2014).
[36]N. S. H. Motlagh, P. Parvin, F. Ghasemi, and F. Atyabi, Biomed. Opt. Express **7**, 2400 (2016).